Chapter 2

CD13/APN in Hematopoetic Cells – Expression, Function, Regulation, and Clinical Aspects

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1. INTRODUCTION

Alanyl aminopeptidase (aminopeptidase N, APN, CD13, EC 3.4.11.2) is a 967 amino acid type II transmembrane protein which occurs on the cell surface of a broad variety of cells. The zinc-dependent metalloprotease (M1 family of peptidases, clan MA, gluzincins) (Hooper 1994, Rawlings and Barrett 1993) preferentially cleaves neutral amino acids from the N-terminus of oligopeptides. The human APN gene was cloned in 1989 and mapped to chromosome 15(q25-q26) (Look *et al* 1989, Watt and Willard 1990). The 3560 bp of coding sequence are scattered over 20 exons (Lerche *et al.* 1996).

The crystal structures of APN or related surface proteases have not yet been resolved. As deduced from its coding sequence (Olsen *et al* 1988), human APN contains seven cysteine residues, all highly conserved between APN from different species. Six cysteines are located in the extracellular part which also contains the active site with the HEXXH motif (amino acids 388 to 392), while only the first cysteine is located in the transmembrane region. In earlier studies it was shown that proper folding of the extracellular part of APN occurs independently of the residual protein (Hussain 1985), suggesting that the transmembrane cysteine 24 does not contribute to structure and function of alanyl aminopeptidase. On the contrary, the importance of cysteine residues in the extracellular part were hardly predictable since. Alanyl aminopeptidase is known to be expressed as a

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homodimer of two non-covalently linked subunits of 150 kDa each (Sjöström and Noren 1982). However, it seems reasonable that disulfide-bridges are involved in the formation of the tertiary structure of alanyl aminopeptidase, especially within its C-terminal domain (Sjöström *et al* 2000).

APN is most strongly expressed in the intestine and in the kidney, and, to a lower extent, also in a number of other tissues (Barnes *et al* 1997, Lucius *et al* 1995). APN gene transcription is initiated at either a myeloid or an epithelial promoter, which are separated from each other by an 8 kb intron (Olsen *et al* 1991, Shapiro *et al* 1991). APN transcripts from myeloid cells are 259 bp longer than those from epithelial cells, but they differ in the non-coding region only. In the myeloid promoter, where transcription is initiated at a set of GC-boxes, members of the Ets and Myb families of transcription factors drive APN expression (Hedge *et al* 1998, Shapiro 1995, Shapiro *et al* 1991, Yang *et al* 1998). The epithelial promoter, containing a regular TATA-box, is located adjacent to the translation initiation site and includes consensus binding sites, for e.g. LF-A1, LF-B1, LF-B2 and LF-C (Olsen *et al* 1995, Olsen *et al* 1991, Shapiro *et al* 1991). An enhancer region of about 300 bp that is positioned 2.7 kb upstream of the epithelial promoter seems to enhance the activity of both promoters (Olsen *et al* 1997).

Within the hematopoietic system, APN is predominantly expressed on cells of the myelo-monocytic lineage. Therefore, anti-CD13 monoclonal antibodies are used as routine markers in the classification of human myeloid leukemias. Mature B and resting T cells lack APN expression detectable by standard flow cytometry. However, APN mRNA could be consistently detected in resting T cells and APN/CD13 expression is markedly up-regulated in response to T cell activation (Lendeckel *et al* 1996, 1997a, 1997b). In addition, both acute (B-ALL) and chronic (B-CLL) B cell leukaemia show abnormal APN surface expression, which in the case of adult B-ALL has been associated with a poor prognosis (Dreno *et al* 1990, Drexler *et al* 1991, Guyotat *et al* 1990, Matsushita *et al* 1994, Pinto *et al* 1991).

The expression of the CD13 antigen on the surface of T cells stimulated by concanavalin A was first reported by Ansorge (Ansorge *et al* 1991) and Kunz and co-workers (Kunz *et al* 1993). Later it was shown by others that a significant fraction of T cells derived from local sites of inflammation is also CD13-positive (Riemann *et al* 1993, 1994). Two recent reviews summarised the general aspects of APN function and expression (Riemann *et al* 1999), of the specific role of T cell APN as well as of the consequences resulting from its inhibition (Lendeckel *et al* 1999).

Alanyl aminopeptidase is supposed to be involved in the degradation of neuropeptides (Ahmad *et al* 1992, Furuhashi *et al* 1988, Giros *et al* 1986,

Miller *et al* 1994a, 1994b, Mizutani *et al* 1993, Shibanoki *et al* 1991, Shimamura *et al* 1988, Shimamura *et al* 1991, Ward *et al* 1990), cytokines and immunomodulatory peptides (Hoffmann *et al* 1993, Kanayama *et al* 1995, Mathe 1987), and angiotensins (Chansel *et al* 1998, Palmieri *et al* 1989, Palmieri *et al* 1985). Furthermore, APN may contribute in extracellular matrix degradation (Fujii *et al* 1995, Saiki *et al* 1993) and antigen processing (Hansen *et al* 1993, Larsen *et al* 1996). APN also functions as a receptor for corona viruses (Delmas *et al* 1992, 1993, 1994, Yeager *et al* 1992) and CMV (Giugni *et al* 1996, Söderberg *et al* 1993). Since most data are obtained from *in vitro* studies, both the physiological ligands and the function of the leukocyte-derived enzyme *in vivo* remain to be established fully.

APN inhibitors of different specificity and, thus, efficacy have been applied in both *in vitro* and *in vivo* studies. Bestatin (Ubenimex), although of rather low specificity, has been most widely used. Bestatin affected the growth of various cell types *in vitro* (Ino *et al* 1991, 1992, Sakurada *et al* 1990). In addition, bestatin inhibited the growth of transplanted tumor cells in a mouse model (Inoi *et al* 1995, Kowalski *et al* 1995) as well as of gastrointestinal tumor or T cell leukemia cells in man (Iwahashi *et al* 1994a, Okamura *et al* 1992, Yamagishi *et al* 1991). Both direct cytotoxic effects (Ino *et al* 1995, Okamura *et al* 1992) and the activation of macrophages, NK cells or cytotoxic T cells (Iwahashi *et al* 1994a, 1994b, Yamagishi *et al* 1991) seem to contribute to these antiproliferative effects. Early reports showed a stimulation by bestatin of the proliferation and DNA-synthesis of human T cells, Concanavalin A-stimulated T-cells, or mouse spleenocytes (Ishizuka *et al* 1980, Müller *et al* 1979, Saito *et al* 1976), but these data are in contrast to those of others (Lendeckel *et al* 1996, 1997b, 1999, Morikawa *et al* 1989). Bestatin, at sub-micromolar concentrations only partially inhibits APN, but shows a significant inhibition of leucyl-aminopeptidase (EC3.4.11.1) and soluble alanyl-aminopeptidase (EC3.4.11.14) (Tieku and Hooper 1992). Actinonin (IC_{50} 2 μM), probestin (IC_{50} 50 nM), phebestin (IC_{50} 20 nM) and RB3014 (IC_{50} 15 nM) appear to be more specific inhibitors of APN (Aoyagi *et al* 1990, Chen *et al* 1999, Nagai *et al* 1997, Tieku and Hooper 1992, Yoshida *et al* 1990), but also inhibited the growth of human T cells, mononuclear cells, and of the T cell lines KARPAS-299 and H9 very effectively (Lendeckel *et al* 1996, 1997b, 1998).

Recent work (Bhagwat *et al* 2001) identified APN as a selective marker of neovascularization and, thus, APN inhibitor application emerged as a powerful anti-angiogenic strategy for the treatment of e.g. cancer. In studies aimed at the identification of peptides that home specifically to solid tumors it was observed that the NGR motif binds to endothelium of angiogenic vasculature (Pasqualini *et al* 1995). Further investigation identified APN,

which is absent from normal vasculature, as the receptor for this peptide motif (Pasqualini *et al* 2000). APN expression of primary endothelial cells and corresponding cell lines is up-regulated in response to hypoxia and angiogenic growth factors (bFGF, VEGF). Of note, angiogenic tube formation was inhibited by exposing endothelial cells to APN inhibitors (Bhagwat *et al* 2001). This subject is dealt with in very detail by Linda Shapiro (this book, chp. 5).

2. EXPRESSION OF APN IN HUMAN T CELLS

Resting T cells were generally regarded as "APN-negative" cells, since they lack CD13 surface expression detectable by standard flow cytometry. However, considerable amounts of APN mRNA could be consistently detected in freshly isolated peripheral T cells by RT-PCR. Significant evidence points to an activation-dependent increase of APN gene and surface expression in response to T cell activation *in vitro* (Ansorge *et al* 1991, Kunz *et al* 1993, Lendeckel *et al* 1996, 1997a, 1997b, 1999, Wex *et al* 1995), which apparently is due to both a stabilization of APN mRNA and an increase of promoter activity (Wex *et al* 1995). In addition, aminopeptidase activity of activated T cells exceeds that of resting cells by 3- to 6-fold (Lendeckel *et al* 1996). Probably as a result of an activation *in vivo*, T cells derived from synovial fluid of patients suffering from rheumatoid arthritis (Riemann *et al* 1993) or from pericardial fluid of patients with various heart diseases (Riemann *et al* 1994) showed significant APN/CD13 expression. A similar induction of APN expression has been observed on tumor-infiltrating T cells (Riemann *et al* 1994).

There are divergent data on the time-course of APN induction. In a co-cultivation model of human tonsillar T cells with synovial cells or endothelial cells, Riemann et al. (Riemann *et al* 1997) observed an induction of APN expression on T cells already 30 minutes after cell-cell contact. This rapid induction of APN-mRNA may relay on the fact that, similar to thymocytes, tonsillar T cells abundently express c-Myb mRNA (Yokota *et al* 1987). In human T cells, we observed maximum APN mRNA levels at days 3 and 4 after activation *in vitro* (Lendeckel *et al* 1996). This finding is in complete agreement with the time-course observed for the mRNA induction of the transcription factor c-Myb, which has been found to play a crucial role in the activation of the myeloid APN promoter (Hedge *et al* 1998). c-Myb has been detected in all leukocyte subsets. Freshly isolated T cells contain minute amounts of c-Myb only, but these levels dramatically increase 48 hours after stimulation by PHA/PMA or anti-CD3 monoclonal antibodies (mab) (Hirai and Sherr 1996, Shipp and Reinherz 1987).

Quantitative RT-PCR revealed that 72 h after activation of T cells by phytohemagglutinine (PHA) and phorbol ester (PMA), amounts of c-Myb mRNA were 60-fold elevated compared to resting T cells (Fig. 1). Most interestingly, this increase of c-Myb mRNA was reduced by 50 % in the presence of the specific APN inhibitor actinonin. This is suggestive of an involvement of APN in early steps of T cell activation.

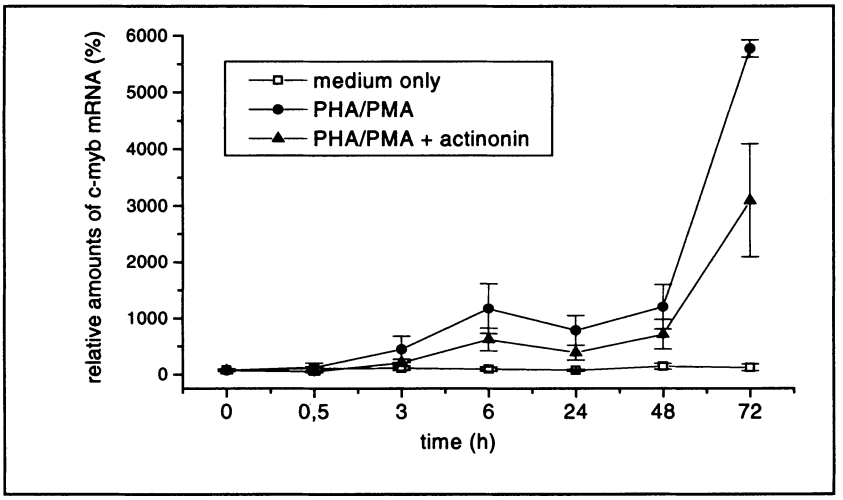

Figure 1: Time-course of the activation-dependent induction of c-myb mRNA in human T cells. T cells were cultured with the additions indicated and then c-myb mRNA amounts were determined by quantitative RT-PCR using the Lightcycler LC24 (Idaho Technology) at the given time points (mean ± sd of three experiments).

Previous work showed that in response to T cell activation there is a bi-phasic induction of CD13 surface expression: a rapid induction which appeared to be independent of *de-novo* biosynthesis was followed by a slower one that required 3 to 4 days to achieve maximum APN mRNA and protein levels (Lendeckel *et al* 1997a). Similarly, stimulation of human leukocytes with anaphylatoxin C5a resulted in a large increase of CD13 surface expression on granulocytes and monocytes within minutes which, in addition, could not be prevented by the inhibitor of protein biosynthesis, cycloheximide (Werfel *et al* 1991). These combined data strongly suggest the existence in granulocytes, monocytes and T cells of an intracellular store of APN that becomes rapidly translocated to the cell surface upon adequate stimulation.

Little is known about APN expression on T cell subsets, a fact that led us to investigate this subject in more detail. First, the major T cell populations, $CD4^+$ and $CD8^+$, were enriched by using the corresponding T cell subset enrichment columns provided by R & D systems. Both populations did not

differ with respect to APN mRNA content, APN enzymatic activity, or CD13 surface expression. This also holds true for activated T cells (not shown).

Second, T cell fractions enriched for either Th1 or Th2 cells were generated by repeated stimulation of peripheral T cells by *Staphylococcus* enterotoxin A (SEA) and IL-2 in the presence of IL-4 or neutralizing anti-IL-4 mab, respectively, over a 10 day culture period. The Th1 fraction, which was characterized by strong expression of the IL-18 receptor (surface expression and mRNA) and IL-2 (mRNA and protein secreted into the culture medium), showed significantly elevated APN mRNA levels and higher APN enzymatic activity, in comparison to the Th2 population (Fig. 2). There was also a trend towards an increased surface expression of CD13 on Th1 cells (3.9 % vs. 1.6 %), but this did not reach statistical significance ($p = 0.11$). Thus, Th1 cells could represent a major target for the beneficial therapeutic application of specific APN inhibitors in Th1-dominated situations such as allograft rejection, chronic inflammation, hypersensitivity, or autoimmune diseases such as multiple sclerosis (see Brocke *et al*, this book, chp. 11) or type I diabetes (Hoffmann and Demuth *et al*, this book, chp. 10).

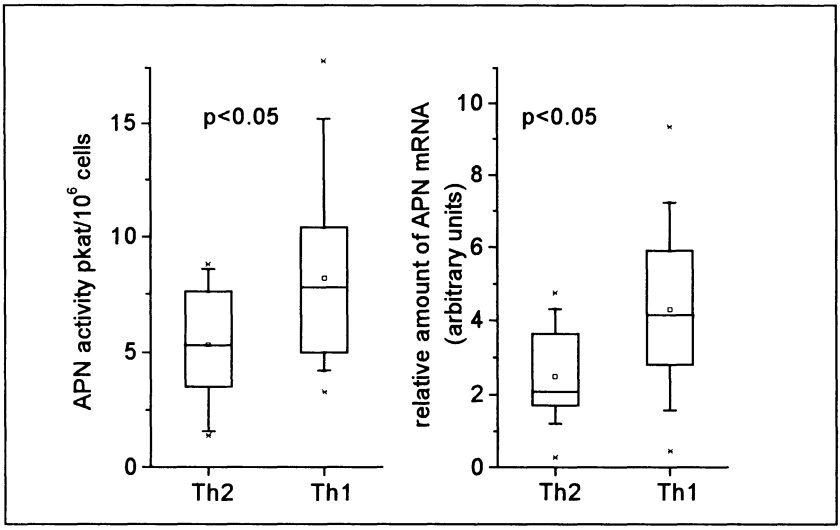

Figure 2: APN enzymatic activity (Ala-pNA-hydrolysing activity which could be fully inhibited by the highly specific APN inhibitor RB3014, 10^{-7} M) (left) and APN mRNA amounts determined by quantitative RT-PCR (right) in enriched fractions of Th1 and Th2 cells (mean ± sd of 16 experiments).

3. APN INHIBITORS AFFECT LEUKOCYTE GROWTH AND FUNCTION

Most studies that made use of APN inhibitors demonstrated their strong anti-proliferative activity towards various cell types, including leukocytes (see above). In our hands, the unspecific aminopeptidase inhibitor bestatin as well as the supposedly more specific APN inhibitors, actinonin and probestin, caused a dose-dependent decrease of DNA synthesis of activated peripheral T cells or mononuclear cells, of the T cell lines KARPAS-299, P12/Ichikawa, H9, and of the pro-myeloid cell line U937 (Lendeckel *et al* 1996, 1998).

The inhibition of APN expression by antisense-oligonucleotides resulted in a similar reduction of DNA synthesis in activated peripheral T cells, H9, KARPAS-299, and U937 cells. This strongly implies that indeed the inhibition of APN caused the growth inhibition observed in response to inhibitor administration. In support of this view we could recently demonstrate that in these cell systems the highly selective and specific APN inhibitor RB3014 also exhibits strong anti-proliferative effects (unpublished).

A fact that needs to be considered is that these growth inhibitory effects could be observed in both $CD13^+$ (U937, Karpas-299, activated T cells) and $CD13^-$ negative cells (H9). Thus, at present it could not be fully excluded that the inhibition of other aminopeptidases contributes to the observed decrease of cellular proliferation. One such candidate aminopeptidase is the ubiquitously expressed soluble alanyl-aminopeptidase (sAAP) (also referred to as puromycin-sensitive aminopeptidase, EC3.4.11.14). This cytosolic aminopeptidase is hardly distinguishable from APN with respect to substrate and inhibitor specificity. In addition, inhibition of sAAP enzymatic activity by puromycin resulted in a growth inhibition similar to that observed in response to decreasing APN expression or activity (Constam *et al* 1995).

It should be kept in mind, however, that "CD13-negative" cells harbour considerable amounts of APN mRNA, which at least in the case of the T cell lines are equal to those of typical $CD13^+$ cells. Therefore, the existence of an intracellular pool of APN has been proposed (Lendeckel *et al* 1997a). In addition, the antisense-mediated decrease of APN expression resulted in growth inhibition in human T cells and T cell lines, as well.

A number of studies addressed the question whether the anti-proliferative effects of APN inhibitors were mediated by changes of cytokine or cytokine receptor expression. Bestatin was reported to increase the concentration of GM-CSF in the culture medium of mononuclear cells (Okamura *et al* 1990) and to stimulate M-CSF receptor expression of U937 cells (Murata *et al* 1994). We could show that the antisense-mediated inhibition of APN

expression in U937 cells increased the amounts of IL-1-receptor antagonist (IL-1RA) detectable in the culture medium, but decreased that of TNF-α (Wex *et al* 1997). Activation of human mononuclear cells led to a rapid increase of IL-1β mRNA amounts within 24 hours, that could be effectively reduced by the aminopeptidase inhibitors actinonin, probestin, and leuhistin. This decrease of IL-1β expression could also be observed at the protein level (unpublished). Probestin was shown to reduce the activation-dependent increase of IL-2 mRNA in human peripheral T cells (Lendeckel *et al* 1999).

A possible key step for mediating the anti-proliferative effects resulting from APN inhibition is the increase of the expression and secretion of the highly potent immunosuppressive cytokine, TGF-β1. Both actinonin and probestin were shown to be capable of inducing TGF-β1 expression in human mononuclear cells (MNC) or peripheral T cells (Lendeckel *et al* 1999). In a number of cell types TGF-β1 leads to a cell cycle arrest in G_1. This is due to an inhibition of expression and/or activity of the cyclin-dependent kinases (CDK) CDK4 and CDK2 (Ewen 1996), which causes a hypophosphorylation of the retinoblastoma protein pRb105 and the formation of complexes of transcription factors such as E2F4-Rb, E2F4-p107, or E2F4-p130 (Li *et al* 1997). E2F-consensus binding sites are crucial elements regulating the TGF-β1-dependent gene transcription. Among the genes controlled by TGF-β1 are those of inhibitors of cyclin-dependent kinases p21/Waf-1 (Dkhissi *et al* 1999, Hunt *et al* 1998), p27/kip (Dkhissi *et al* 1999, Mahmud *et al* 1999), and p15/INK4B (Ewen 1996, Li *et al* 1997), as well as the activator of G_1-CDKs, cdc25A (Iavarone and Massague 1998), and the anti-apoptotic bcl-2 (Mahmud *et al* 1999). Besides the E2F family of transcription factors, Smad4 and DPC4 were identified as essential mediators of TGF-β1 (Hunt *et al* 1998). As demonstrated by gel-shift analysis, RB3014 provoked an induction of the transcription factor Sp1 in human peripheral T cells (Fig. 3). Whereas Sp1-site binding protein was not detectable in nuclei of peripheral T cells that were allowed to rest for 24 hours in RPMI 1640, supplemented with 10 % fetal calf serum, it was easily detectable 30 and 60 mins after administration of the inhibitor. Notably, the genes of both p21/Waf-1 and TGF-β1 itself are responsive to Sp1 (Geiser *et al* 1993, Han *et al* 2001, Koutsodontis *et al* 2001, Udvadia *et al* 1993). These results provide a reasonable mechanism how APN inhibition might interfere with the cell cycle machinery. Considering the fact that there has been observed a specific, "disease-associated" induction of APN expression and activity in T cells or endothelial cells, this "abnormally" expressed APN represents a promising target for the therapeutic application of APN inhibitors that could induce the production and release of TGF-β1 from these cells locally restricted at the site of their occurrence.

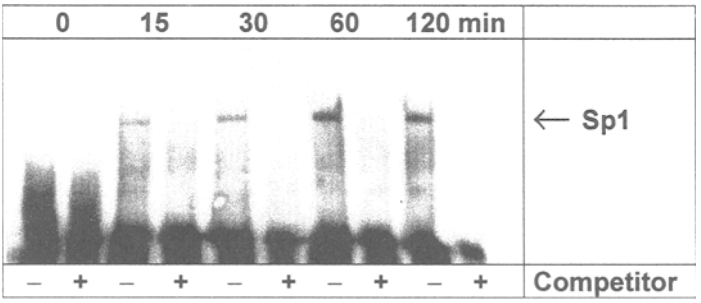

Figure 3: Gel-shift assay of human peripheral T cell nuclear protein binding to Sp1 consensus sites. Whereas no Sp1-binding factor is detectable in untreated T cells, it was induced in response to the APN inhibitor RB3014 within 15 min. Maximum levels were detected 60 min after inhibitor administration. (competitor: 100fold excess of unlabeled Sp1 oligonucleotide).

4. MECHANISMS OF APN SIGNALING

A few ectopeptidases present on leukocyte surfaces are supposed to be capable of inducing or modulating signal transduction (for review see Goding and Howard 1998, Riemann *et al,* this book, chp. 6). Emerging evidence attributes a "signalling" function to APN/CD13 as well. Bestatin changed activity and cellular localization of PKC in K562 cells (Kumano and Sugawara 1992). In U937 cells, bestatin increased the activity of the Pp60/c-Src tyrosin-kinase (Murata *et al* 1994). A very recent study aimed at the identification of potential targets mediating the anti-proliferative effects of alanyl-aminopeptidase expression and activity revealed a modulation of MAP kinase p42/Erk2 activity and mRNA-levels in KARPAS-299 cells by probestin and actinonin (Lendeckel *et al* 1998). Both inhibitors at 50 µM concentration caused a maximum 3.5fold elevation of erk2-mRNA content after 24 hours, compared to KARPAS-299 cells cultured in medium only. Notably, maximum activation (phosphorylation) of p42/Erk2 was observed 8 hours after inhibitor administration. Actinonin provoked a 7fold increase of Pp42, whereas probestin caused a 3fold increase only. From the different time courses observed for p42/Erk2-activation and its mRNA induction it must be concluded that both mechanisms function independently of each other.

A phosphorylation (activation) of Erk1/2 could also be observed after ligation of APN/CD13 by anti-CD13 mab in human monocytes (Santos *et al* 2000).

Subsequently, by applying cDNA array and quantitative RT-PCR technique a upregulation of Wnt-5a expression by actinonin was shown in peripheral human T cells (Lendeckel *et al* 2000a). Furthermore, expression and activity of glycogen-synthase kinase-3β (GSK-3β), an inherent component of the Wnt-signaling pathway, were found to be increased in the course of T cell activation (Lendeckel *et al* 2000b). These changes were not restricted to special T cell mitogens, but rather depend on an effective T cell activation. This view is supported by the observation that both PHA and pokeweed mitogen (PWM) as well as combinations of anti-CD3 mab with IL-4 or IL-9, respectively, were capable of inducing GSK-3β mRNA and protein expression. Several APN inhibitors, including the highly specific compound RB3014 partially reversed these activation-dependent changes of GSK-3β expression and activity (illustrated in Fig. 4).

Wnt-signals increase the phosphorylation of GSK-3β at Ser9 and, thereby inactivate this negative regulatory protein kinase. This prevents substrates such as β-catenin, eIF2B or cyclin D1 from being phosphorylated and, thus, controls their activity or rescues them from proteasome-mediated degradation (Aberle *et al* 1997, Diehl *et al* 1998). Consequently, there is a stimulation of e.g. TCF-mediated transcription, protein biosynthesis, and cell cycle progression. In addition to the regulation by Wnt-5a, GSK-3β activity is regulated by PKB, which itself is controlled or modulated by e.g. MAP kinases, PKA, PI3-kinase and, interestingly, signaling from the T cell co-stimulatory molecule CD28.

Inhibition of GSK-3β by Li^+ caused a sustained cell cycle arrest at G2/M transition in endothelial cells without compromising cell viability (Mao *et al* 2001). Interestingly, Li^+ increased the expression of the CDK inhibitor, p21, at both the mRNA and protein levels. This induction of p21 was clearly dependent on the presence of p53, which is indicative of an activation by GSK-3β of also the p53 pathway. Remarkably, an inhibitor of methionyl aminopeptidase I, TNP-470, provokes cell cycle arrest of endothelial cells in late G_1, which also appeared to depend on the presence and activity of p21 and p53 (Yeh *et al* 2000).

On the contrary, however, in human T cells the antigen-specific activation decreased GSK-3β activity and its inhibition by Li^+ prolonged the proliferative response *in vitro* (Ohteki *et al* 2000). In complete accordance with these data, we also observed an increase of phosphorylated GSK-3β in response to mitogenic T cell activation.

The recent work of Hoeflich et al. (2000) identified NF-κB as a substrate of GSK-3β. Therefore, a role of GSK-3β in the regulation of (a subset of) survival genes, typically triggered by the TNF-α pathway, is suggested. This mechanism may account for the lack of cytotoxicity of APN inhibitors.

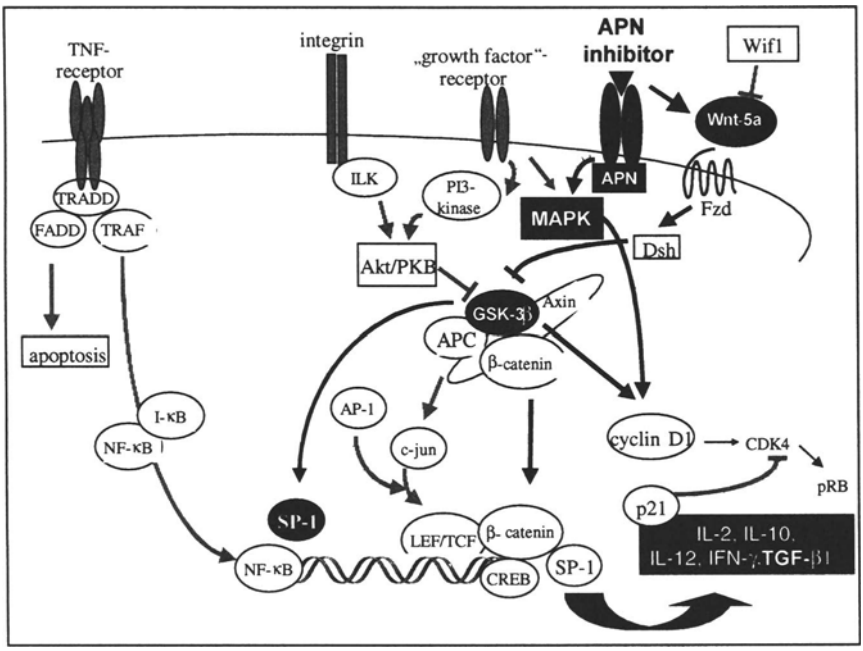

Figure 4. Supposed coupling of APN to cellular signalling pathways of human T cells. Inhibition of APN leads to an activation of MAP kinases Erk1/Erk2 and increases Wnt-5a expression. In addition, the activation-dependent phosphorylation of GSK-3β is decreased by the application of APN inhibitors. The induction of Sp1 transcription factor together with the altered activity of e.g. NF-κB, β-catenin and members of the LEF/TCF family of transcription factors could mediate changes in proliferation rate and cytokine expression (TGF-β1). Components possibly involved in APN-signalling are given on black background. For abbreviations see text.

The observed increase of GSK-3β expression and its simultaneous phosphorylation/inactivation requires further investigation. As we analyzed a rather late than an early phase of T cell activation, this GSK-3β induction might represent mechanisms already limiting activation. As shown by Thomas et al. (1999), substrate specificity may depend on a „priming" phosphorylation, not necessarily required in later states of activation. Finally, cellular amounts in activated T cells of active GSK-3β may exceed those of resting cells anyway.

Further support for the idea that APN is capable of triggering signal transduction events comes from the observation of Löhn *et al* (1997) who detected a transient but weak increase in intracellular Ca^{2+}-levels after exposing U937 cells to anti-CD13 mabs. The possible coupling of APN to ion channels needs further investigation.

5. MUTATIONS IN THE HUMAN APN GENE

A significant fraction of malignant lymphocytes and corresponding cell lines appear CD13-positive in flow cytometry. In addition, "CD13-negative" T and B cell lines were shown to contain considerable amounts of APN mRNA (Lendeckel *et al* 1996). Therefore, it was suggested that a dysregulation of APN expression contributes to or results from malignant transformation of lymphocytes and/or cellular growth.

The human APN gene lacks extensive polymorphisms and until recently DraI, DraIII and BclI polymorphisms were the only definite reports on mutations in that gene (Watt and Willard 1990, Kruse *et al* 1992). A family with inherited (autosomal dominant) increased serum APN activity has been reported recently (Kawai *et al* 1998). Further investigation of these cases revealed, however, that there were no mutations in the APN cDNA detectable (Kawai *et al* 2001). In serum of this family, a novel truncated form of the CD13 polypeptide lacking amino acids (aa) 1-43 was found in high concentrations. In contrast, serum from healthy volunteers predominantly contained CD13 lacking aa 1-58 (Kawai *et al* 2001, Watanabe *et al* 1995). It is suggested, therefore, that elevated levels of truncated APN are due to a mutation in a gene that regulates 43-truncation proteolytic activity. The protease catalyzing 43- or 58-truncation of CD13 may play an important role in several pathophysiological mechanisms through down-regulation of cell surface APN.

Two splice variants in which exons 3 and exon 14 were lost were identified by Dybkaer et al. (2001). These splice variants made up not more than 10 % of the APN mRNA in healthy individuals and the majority of acute myeloid leukemia (AML) patients. Increased expression of both truncated forms was observed in 6 % of AML cases, whereas no detectable exon 3 or exon 14 splice variants were found in 26 % and 9 % of AML patients, respectively.

Similarly, by applying RFLP and SSCP analysis, mutations in the APN gene could be detected in 18 % or 6 % of cases, resp., of leukemia/ lymphoma (Lendeckel *et al* 1998). No such mutations were found among healthy controls. Cloning and sequencing of mutant APN-cDNA fragments revealed the presence of "silent" mutations not expected to affect protein structure and function, the introduction of stop-codons at amino acid (aa) 230 or 232, respectively, and the deletion (Δ227Pro) or substitution (Leu243Pro) of single aa residues. The effects of the latter 2 mutations on APN surface expression and enzymatic activity were studied in detail. At first, the APN cDNA from U937 cells was cloned into the pEGFP-N1 vector (Clontech), resulting in the expression vector (pEGFP-APN) containing enhanced GFP fused in frame to the 3' end of wild type APN. The mutations

Δ227Pro and Leu243Pro were introduced into pEGFP-APN by site-directed mutagenesis using the QuickChange™ site-directed mutagenesis kit from Stratagene. The constructed vectors pEGFP-APN-Δ227Pro and -Leu243Pro were transiently transfected into EcR-293 cells using the Effectene™ transfection reagent (QIAGEN) and cells were assayed after 2 days of culture.

The C-terminal attachment of GFP to wild type APN did not change either its subcellular localization or its function: the typical surface expression of wild type APN was completely retained as could be shown by fluorescence light microscopy (Fig. 5A). This implies that protein folding, glycosylation pattern, and intracellular transport were not markedly influenced. Results of cytofluorimetric analyses confirmed intense surface expression of APN-GFP when using either anti-GFP or anti-CD13 antibodies. In contrast, the mutations provoked changes in the expression pattern of the APN-GFP fusion proteins, with apparently predominant intracellular localization (Fig. 5C, D). This was confirmed by cytofluorimetric analysis using an anti-GFP antibody. A clear-cut surface expression was only observed in cells transfected with wild type APN-GFP (27 % positive cells, mean fluorescence intensity (mfi) of 200.2, compared to less than 4 % positive cells and mfi values of less than 45 for the mutants).

Therefore, detailed co-localization studies were applied to address the subcellular sites of accumulation. A clear overlap of the GFP-derived fluorescence with an organelle specific marker could only be observed in the case of the ER-staining with ER-Tracker Blue-White DPX. The obtained fluorescence showed a typical ER staining pattern around non-fluorescent cell nuclei. Using BODIPY TR ceramide as a trans-Golgi probe, a staining pattern obviously different from the ER-staining could be observed (not shown).

To confirm these results, analysis of endoglycosidase H sensitivity and PNGase F treatment were carried out for all APN-GFP fusion proteins. Treatment with endoglycosidase H allows distinguishing high mannose (sensitive) from complex (insensitive) sugars, and thus, deciding whether a protein was able to leave the ER and to reach the Golgi. PNGase F digestion allows one to proof whether different protein forms are due to variations in the glycosylation pattern.

Immunoblot analysis using an anti-GFP antibody identified two bands of approximately 170 and 190 kDa in the case of wild type APN-GFP. The band with the higher molecular weight is in full accordance with the 166 kDa monomeric form of mature APN enlarged by 27 kDa of monomeric GFP. The detected double band represents different glycosylation forms, as confirmed by PNGase F treatment. Endoglycosidase H digestion resulted in

degradation of the lower molecular weight species, indicating this being a mannose-rich glycosylation form of APN (Fig. 5B, 6).

The PNGase F digestion resulted in the formation of one single band of 140 kDa in all cases, which obviously represents the unglycosylated form of APN (115 kDa), enlarged by the monomeric GFP (27 kDa). Corresponding to the results for wild type protein, the double band detected in the case of the mutants is reduced to one single band after PNGase F treatment, suggesting that two different glycosylation forms are present in the particulate fraction (Fig. 6).

Treatment with endoglycosidase H revealed the presence of endoglycosidase H-sensitive proteins in particulate fractions of wild type and mutant APN-GFP. One additional insensitive band of higher molecular weight, corresponding to the complex glycosylated mature form of APN transported to the cell surface, is only found for wild type APN. In contrast, the GFP fusion protein is completely sensitve to endoglycosidase H in the cases of APN mutants (Fig. 6), confirming the results of the co-localization studies, which showed a clear ER-retardation of these proteins.

Furthermore, the neutral aminopeptidase activity of the wild type enzyme was fully preserved in APN-GFP. The Ala-pNA hydrolyzing activity of the particulate fraction of EcR-293 cells was significantly increased upon transient transfection with pEGFP-APN in comparison to non-transfected cells showing only 47.7 % of the activity of cells transfected with wild type APN-GFP. The mutants Δ227Pro and L243Pro showed markedly decreased specific activities of less than 42 % of wild type activity in all cases ($p < 0.05$), representing a decrease of wild type activity back to the control level (48 %) (Fig. 7).

The results of this study clearly show that the deletion of the proline residue 227 from APN (Δ227P) and the substitution of proline for Leu243, as observed in patients suffering from acute lymphatic leukemia of B cell type (B-ALL) or non-Hodgkin lymphoma (NHL) (Lendeckel *et al* 1998) result in a completely enzymatically inactive APN. Furthermore, dramatic changes in the expression pattern of these mutants were observed in comparison to the wild type protein. Proper folding of a protein is a prerequisite for its translocation from ER to Golgi, and misfolded or misassembled proteins are retained in the ER or degraded (Klausner and Sitia 1990).

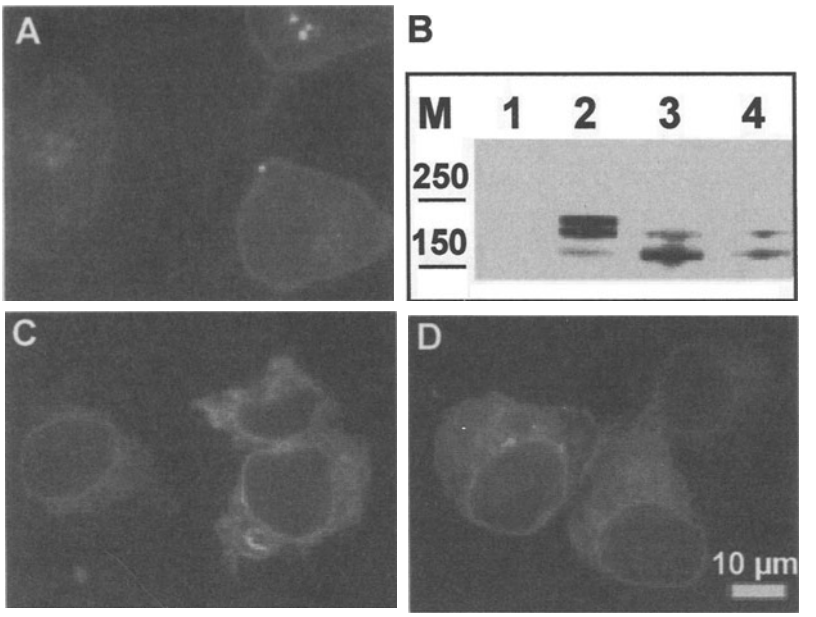

Figure 5: Cellular localization (A, C, D) and immunoblot analysis (B) of wild type and mutant APN-GFP fusion proteins expressed in EcR-293 cells. Wild type APN shows typical membrane expression (A), whereas the Δ227Pro (C) and Leu243Pro (D) mutants are retained obviously in the ER. B: Immunoblot of the particulate fraction of EcR-293 cells transfected with control plasmid (lane 1), wild type APN-GFP (lane 2), the APN-GFP mutants Δ227Pro (lane 3) and Leu243Pro (lane 4). APN proteins were detected by means of anti-GFP antibodies (Living colors peptide antibody; Clontech). M = molecular weight marker

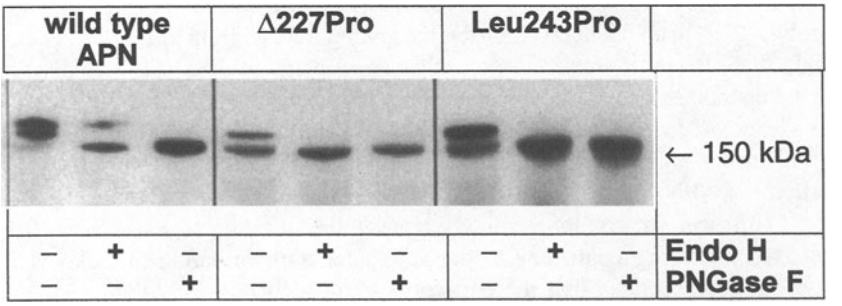

Figure 6: Analysis of the glycosylation of wild type and mutant APN-GFP fusion proteins. The particulate fractions of transiently transfected EcR-293 cells were incubated in the presence (+) or absence (–) of PNGase F or endoglycosidase H and then separated on a 4 - 12 % denaturing gradient polyacrylamid gel. APN proteins were detected by means of anti-GFP antibodies (Living colors peptide antibody; Clontech).

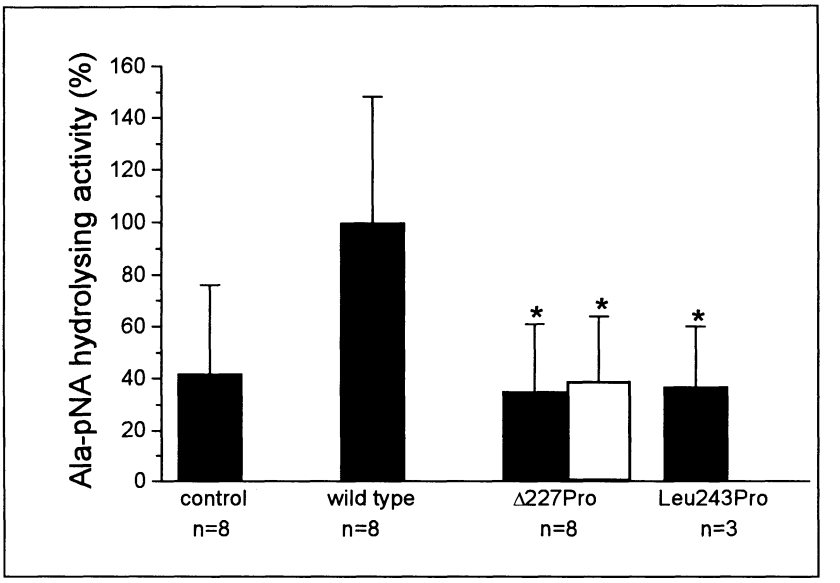

Figure 7: Relative APN enzymatic activity of the membrane fraction of EcR-293 cells transiently transfected with wild type or mutant APN-GFP fusion proteins, or untransfected cells (control). (mean ± sd, wild type activity set as 100 %, *$p<0.05$). Black and white boxes represent independent clones.

Proline generally restricts the conformation of the peptide bond and, thus, has great impact on protein conformation and function (MacArthur and Thornton 1991). P227 is localized in a highly conserved predicted loop region ranging from amino acids 216 to 227. Based on the fact that the proline 227 itself is highly conserved among APNs from different species and that is even retained in other members of the M1 family of metallopeptidases, a special function has been proposed for this region and, in particular, to proline 227 (Sjöström *et al* 2000). Our data support the idea of Sjöström and colleagues that this region is essential for the enzymatic activity of APN.

At present we cannot definitely answer the question to what extent the detected mutations contribute to the malignant transformation of leukocytes. It could be speculated that these mutations cause changes of APN enzymatic activity and/or stability of APN mRNA and thereby play a role in the multistep process of oncogenesis, especially within the hematopoietic system. In support of this view no such mutations were found among healthy controls.

As described above, members of the Ets and Myb families of transcription factors are important regulators of APN gene transcription.

Interestingly, a mutation in the transactivating domain of Ets-1 has been reported in a case of acute T cell leukaemia (Collyn d'Hooghe *et al* 1997). Ets-1, which is strongly expressed in resting T cells, becomes down-regulated upon T cell activation (Romano-Sica *et al* 1995). Ets-1 seems to repress the activity of the APN promoter. Therefore, loss of Ets-1 binding to the APN promoter could enhance both APN expression and the malignant phenotype. The expression of Ets-2 appears to be reciprocal to that of Ets-1 (Bhat *et al* 1990). The human ets-2 gene has been mapped to the minimal Down syndrome region on chromosome 21 (Papas *et al* 1990) and translocations involving this region, e.g. t(8;21), change the ets-2 gene dosage and thereby may contribute to an increased risk for the development of leukaemia. In this way, changes of transcription factor expression may alter the expression of target genes such as APN.

6. CONCLUSIONS

The data discussed here support the hypothesis that appropriate expression and enzymatic activity of APN/CD13 on cells of the immune system plays a role in the regulation and/or modulation of leukocyte growth and function. Accumulating evidence also suggests the capability of special (patho)-physiological conditions such as malignant transformation, inflammation, T-cell activation, autoimmune disease, and allograft rejection of inducing APN/CD13 expression in human T lymphocytes. Thereby, T cells emerged as targets for an alternative therapy of both T cell-mediated or T cell-dominated diseases, especially, as our data implicate, of the Th1 type. As explained in detail by Linda Shapiro (this book, chp. 5), APN expressed exclusively on endothelial cells of angiogenic microvasculature appeared as a highly promising target for anti-tumour therapy. Of note, inhibition of APN also suppressed the invasion activity of human prostate and renal cancer cell lines (Ishii *et al* 2001a,b).

Although our understanding of the molecular and cellular mechanisms that mediate the anti-proliferative and immunosuppressive effects resulting from APN inhibition is still in the very beginning, the observed modulation of GSK-3β activity and TGF-β1 expression could represent essential key steps.

Potent and selective inhibitors of APN became available during the last years, and these did show neither cytotoxic activity nor other side effects in both *in vitro* and *in vivo* settings, making them suitable pharmcological tools.

Future studies, besides aiming at the identification of the molecular and cellular mechanisms of action of APN inhibitors, will focus on the specific targeting of APN inhibitors to the sites of their request (e.g. neovasculature

or local inflammatory site). Furthermore, the most promising indications for a successful APN inhibitor therapy will be defined by using specific animal models of diseases.

Of note, inhibiting the enzymatic activity of another leukocyte ectopeptidase, namely dipeptidylpeptidase IV (DPIV, CD26), also suppressed growth and function of immune cells, including T cells. Therefore, DPIV inhibitors gained interest as potential immunosuppressive compounds as well (see: Kähne *et al*, this book, chp. 8; Brocke *et al*, this book, chp. 11 and Hoffmann and Demuth *et al*, this book, chp. 10).

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